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RAPID COMMUNICATION

A Mechanism of Resistance to HIV-1 Entry: Inefficient Interactions of CXCR4 with CD4 and gp120 in Macrophages

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To test the hypothesis that inefficient interactions of CXCR4 with CD4 and gp120 could affect HIV-1 entry, we incubated macrophages, monocytes, and lymphocytes with gp120 and coimmunoprecipitated CD4 by using anti-CXCR4 antibodies. CD4 was efficiently coimmunoprecipitated in lymphocytes and monocytes but not in macrophages. Overexpression of CD4 in macrophages resulted in detection of CD4–CXCR4 and gp120–CD4–CXCR4 complexes in parallel with the restoration of macrophage fusion susceptibility. These results suggest a mechanism of resistance to entry of some X4 HIV-1 strains into macrophages and a method for dissection of the initial stages of HIV entry.

Key Words: HIV-1; CD4; CXCR4; coreceptors; chemokine receptors; envelope glycoprotein; membrane fusion.

Introduction. It has been known for many years that T cell line adapted (TCLA) X4 HIV-1 strains do not infect macrophages as efficiently as $CD4^+$ T cells (reviewed in (*1*)). Although the infection could be restricted at a postentry step (*2*), the TCLA X4 HIV-1 envelope glycoproteins (Envs) cannot mediate efficient fusion of macrophages (*3*), suggesting that the entry step is also affected. Based on our previous observations that CXCR4 forms a complex with CD4 and gp120 from TCLA X4 HIV-1 in cell lines susceptible to HIV-1 entry (*4, 5*) we hypothesized that the mechanism of resistance to HIV-1 entry in macrophages could be caused by inefficient interactions of CXCR4 with CD4 and the CD4–gp120 complex (*1, 6*). Here we present data supporting this hypothesis, provide a method for dissection of the initial stage of HIV-1 entry, and propose that inefficient interactions of HIV coreceptors with CD4 and gp120 could play a role in the resistance to HIV entry into a variety of cells.

Results and Discussion. We have examined the abilities of prototypic TCLA X4 and R5 HIV-1 Envs (*7*) to mediate cell fusion in macrophages, while modulating the expressed levels of CD4 and/or CXCR4 or CCR5, using a well-characterized reporter-gene assay for HIV Env-mediated fusion (*3*). Under conditions of wild-type CD4 and coreceptor levels, HeLa cells expressing the TCLA X4 Env LAV were very limited in the ability to mediate fusion with macrophages (Table 1). However, they adequately supported fusion by the R5 Envs JRFL and ADA (Table 1) (*3*), although the surface concentration of CCR5 was about the same as or lower than that of CXCR4 (Table 2). Upon high cell surface CD4 levels (on the order of 10^5 molecules per cell), the LAV Env-mediated fusion increased from background levels (comparable to those given by negative controls) to levels in the range of some R5 Envs (JRFL, ADA) without overexpressed CD4 (Table 1 and data not shown). Overexpression of CXCR4 increased fusion, although the increase was never as high as that when CD4 was overexpressed (Table 1 and data not shown). We also found that simultaneous overexpression of CD4 and CXCR4 consistently resulted in a greater than additive increase in X4 Env-mediated fusion in macrophages. Overexpression of CCR5 or CD4 plus CCR5 also led to significant increase in R5 Env-mediated fusion. These data indicate that efficient fusion mediated by LAV Env results only through the elevation of receptor levels, supposedly when adequate complex formation can occur between CXCR4, CD4, and gp120.

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TABLE 1

HIV-1 Env-Mediated Fusion Measured by a Vaccinia Virus-Based Cell–Cell Fusion Assay

Env/Rec	No target cells	WR	CD4	CXCR4	CCR5	CD4 + CXCR4	CD4 + CCR5
No Env	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.4	0.4 ± 0	0.7 ± 0.4	0.2 ± 0	0.1 ± 0.1
LAV	0.4 ± 0	1.0 ± 0.4	6 ± 1	2.9 ± 0.7	0.8 ± 0.3	40 ± 10	9 ± 3
JRFL	0.2 ± 0	14 ± 3	69 ± 18	17 ± 5	27 ± 11	100 ± 2	230 ± 80
ADA	0.2 ± 0	22 ± 4	91 ± 7	22 ± 2	56 ± 12	92 ± 35	176 ± 41

Note. Macrophages were infected with vaccinia viruses encoding CD4, CXCR4, or CCR5 (control wild-type vaccinia (WR), vCB3 (encoding CD4), vCBYF1 (encoding CXCR4), vvCCR5-1107 (encoding CCR5)) or with combinations of two viruses and with vTF7-3 (encoding T7 RNA polymerase) and incubated overnight (12 h) at 31°C. Effector (HeLa) cells were infected with vaccinia viruses encoding an X4 HIV-1 Env (LAV) or R5 Env (JRFL, ADA) and reporter virus vCB21R–LacZ encoding the *Escherichia coli* LacZ gene linked to the T7 promoter. The two populations of cells were mixed at a ratio of 1:1 (total number of cells equaling 2×10^5 in 96-well plate format in duplicate). Fusion was allowed to proceed for 2 h and quantitated by a colorimetric assay of β-galactosidase activity in detergent cell lysates. No Env indicates infection with only vCB21R–LacZ; no target cells, only infected HeLa cells were measured; the numbers are the mean and standard deviation of the optical density readings and are proportional to the number of fusion events.

To test the hypothesis that the mechanism of resistance to Env-mediated membrane fusion of macrophages could be caused by inefficient interactions between CXCR4, CD4, and gp120, CXCR4-expressing monocytes, lymphocytes, and macrophages were incubated with HIV-1 gp120 (IIIB/BH8) and CD4 was coimmunoprecipitated by using anti-CXCR4 monoclonal antibodies (mAbs) (Fig. 1). CD4 was efficiently coimmunoprecipitated in monocytes and lymphocytes but not in macrophages (Fig. 1), while CD4 coimmunoprecipitated with anti-CCR5 antibodies can be readily detected under similar conditions even in the absence of gp120 (Fig. 1) (*5*). Upon high CD4 expression, which allows for membrane fusion of the macrophages with LAV Env, we were able to coimmunoprecipitate the CD4–CXCR4 complex (Fig. 2). The amount of CD4–CXCR4 complex detected was enhanced upon the inclusion of soluble gp120, as previously observed for coimmunoprecipitates from cell lines (*4, 5*). In fact, the level of CD4–CXCR4 or gp120–CD4–CXCR4 complexes increased, as did the cell fusion, upon overexpression of both CD4 and CXCR4 (with or without gp120, Fig. 2, lanes 5 and 6, respectively). Similar results were obtained for macrophages from several different donors (data not shown). These findings provide a biochemical correlate to the notion that the resistance to TCLA X4 HIV-1 entry in macrophages is due to the inability of these cells to efficiently form complexes between CD4, CXCR4, and gp120 and provide direct evidence of a correlation among the cell surface concentrations of CD4–CXCR4 complexes, gp120–CD4–CXCR4 complexes, and Env-mediated membrane fusion.

TABLE 2

Flow Cytometry Analysis of Macrophages Stained with Anti-CXCR4 mAbs and the Anti-CCR5 mAb m182

Abs	CXCR4 (4G10)	CXCR4 (12G5)	CCR5 (m182)	CD4 (OKT4)
PE molecules	12,000	18,000	12,000	5000

Note. The numbers represent the number of PE molecules bound to the macrophage surface where the background is subtracted and are approximately equal to the number of receptor molecules at the macrophage surface.

These results are important not only because they suggest a likely and possibly universal mechanism explaining a number of observations that expression of CD4 and HIV coreceptors is not sufficient for HIV entry into certain cells, particularly macrophages, but also because they directly demonstrate a correlation between the ability of an X4 HIV-1 Env to mediate membrane fusion and the formation of the gp120–CD4–CXCR4 complex as an initial stage of HIV-1 entry. Such a mechanism has been proposed by several investigators (*4, 8–11*) but had not been formally experimentally demonstrated. While this paper was under review, another independent study suggested that CD4–CXCR4 association is critical for X4 Env-mediated fusion in macrophages and the reduced activities of X4 isolate Env interaction were due to poor complex formation with a high molecular weight species of CXCR4 (*12*). However, our report differs in several significant respects. One critical distinction is that we cannot detect CD4–CXCR4 complexes in either monocytes or macrophages without the addition of Env, and when added we immunoprecipitate gp120–CD4–CXCR4 complexes only from monocytes and lymphocytes, but not from macrophages. In addition, we also correlate the restoration of fusion to the appearance of gp120–CD4–CXCR4 trimolecular complex formation, and we demonstrate the penultimate enhancement of X4 Env-mediated fusion in macrophages through the simultaneous overexpression of both CD4 and CXCR4.

Interestingly, it appears that the formation of the CD4–CXCR4 complex is enhanced by the constitutive association between CD4 and CXCR4 even in the absence of gp120 (Fig. 2) achieved by receptor overexpression. Al-

though the association of CXCR4 with CD4 is much weaker than the CCR5–CD4 interaction (*5*), the CD4–CXCR4 complex still can serve as a nucleus for the subsequent formation of multimeric HIV Env–CD4–CXCR4 complexes leading to formation of fusion pores and fusion. This is supported by our finding that the number of CD4–CXCR4 complexes correlates with both the number of gp120–CD4–CXCR4 complexes and the extent of fusion. Our results are in agreement with the work of Kabat and co-workers (*13*), where R5 primary isolates were shown to have a CD4 and CCR5 interdependent relationship for fusion and entry, and it was proposed that CD4 and CCR5 might directly interact. Regarding primary macrophages where CCR5 is in excess of CD4 we also note that the elevation of CD4 and not CCR5 had the greater effect in fusion enhancement. Further, it was earlier concluded that some primary patient-derived X4 isolates would bind weakly to CD4 and preferentially infect cells that coexpress CXCR4 and large amounts of CD4 (*14*). Our results with macrophages support this notion as well and suggest that the variable results of X4 isolate infection or fusion in human macrophages, whether they be primary or lab-adapted strains, is the result of a particular isolate's ability to orchestrate an adequate number of Env–CD4–CXCR4 complexes, perhaps mediated through a mechanism of differential affinities for the two receptor proteins.

What causes the inefficient interaction of macrophage CXCR4 with CD4 and the CD4–gp120 complex is presently unclear. Previously, we speculated on the possibility that the interaction of CD4 with CCR5 is stronger than that with CXCR4, leading to larger numbers of CD4–CCR5 complexes than CD4–CXCR4 complexes and that this may be a significant factor in the observed differential susceptibility of cells, including macrophages, to entry of X4 and R5 HIV isolates, especially at low levels of CD4 (*1, 6, 15*). However, we recently demonstrated that for some U937 cell clones (minus clones), which resist HIV-1 entry and behave similarly to macrophages but do not express CCR5 (*16*), CXCR4 also does not efficiently

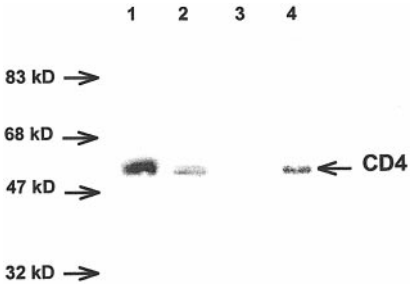

FIG. 1. Coimmoprecipitation of CD4 induced by gp120 in monocytes, lymphocytes, and macrophages. Lanes 1–3 represent CD4 Western blots of immunoprecipitates (by the anti-CXCR4 mAb 4G10) from monocytes, lymphocytes, and macrophages, respectively, incubated with 10 μg/ml gp120. Lane 4 represents a CD4 Western blot of macrophage lysates immunoprecipitated by the anti-CCR5 mAb 5C7.

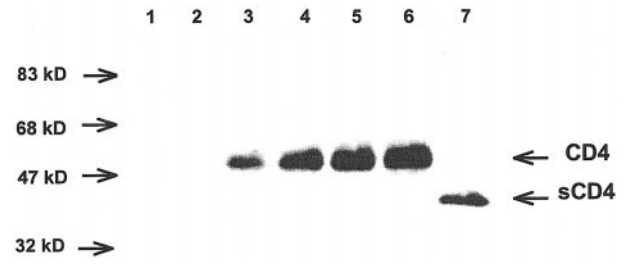

FIG. 2. Immunoprecipitation of CD4–CXCR4 and gp120–CD4–CXCR4 complexes. Lane 1, macrophages infected with wild-type vaccinia virus without gp120; lane 2, as in lane 1 but with gp120; lane 3, overexpression of CD4 by vaccinia without gp120; lane 4, as in lane 3 but with gp120; lane 5, simultaneous infection with vaccinia viruses encoding the genes for CD4 and CXCR4 without gp120; lane 6, as in lane 5 but with gp120; and lane 7, 1 ng of sCD4 (10^{10} molecules) as a quantitative marker.

interact with CD4 and the CD4–gp120 complex (Xiao *et al.,* submitted for publication). Further, we have demonstrated a constitutive association of CD4 and CCR5 in a variety of cells even in the absence of HIV gp120 (*5, 37*). The fact that the anti-CXCR4 mAb 12G5 has been demonstrated to differentially inhibit HIV-1 infection in both a cell type and virus strain dependent manner (*17*) has suggested another possibility—that the CXCR4 molecule itself is somehow posttranslationally differentially processed in macrophages, resulting in its being differentially utilized by various HIV-1 isolates (*18*). A modified CXCR4 protein may have a weaker affinity or ability to associate with CD4 in macrophages or be utilized only by certain primary X4 or R5X4 HIV-1 isolates possessing Envs capable of circumventing this restriction, perhaps through a different binding interaction. Such a possibility is supported by our recent observation that CXCR4 N–linked glycosylation has a profound influence on CXCR4 coreceptor activity (Chabot *et al.,* unpublished data). Indeed, it has been proposed that a different molecular weight species is evident in macrophages which cannot form complexes with CD4 (*12*). However, we found that for both macrophages (data not shown) and U937 minus cells (*37*) CXCR4 is not detectable by Western blotting using an anti-CXCR4 mAb or several rabbit polyclonal serums, all of which otherwise are very efficient in detecting CXCR4 in T lymphocytes or recombinant expressed CXCR4. Thus, at this time we cannot confirm the presence of a high molecular weight form of CXCR4 in macrophages and attribute this discrepancy to different reagents and/or assay sensitivities.

These data not only detail a mechanism of macrophage resistance to infection by HIV-1 but also suggest a possibility for a general mechanism of resistance to HIV-1 entry into a variety of cells. We have also not overlooked a potential implication of these results regarding HIV vaccine development. That is, our findings demonstrate a correctable, measurable biochemical and functional defect in the ability of a TCLA X4 isolate Env

glycoprotein to associate with receptors and induce fusion of primary macrophages, and this suggests that such Envs are indeed structurally and functionally distinct from primary isolate X4 or R5X4 Envs, which further questions their utility as an appropriate molecule in the development of HIV vaccines. Thus, understanding the details of the gp120–CD4–coreceptor interaction is of particular significance for defining further, or to perhaps enhance the presentation of, the conserved and only transiently exposed epitopes or neoepitopes that may be important for eliciting broadly effective anti-HIV Env-based humoral response (*19, 20*).

It has been recently demonstrated that some primary X4 HIV-1 strains are able to enter macrophages (*21, 22, 22a*) and that an X4 HIV strain can cause a rapid decline of CD4 cells in individuals who are homozygous for the 32-bp deletion in the gene coding for CCR5 (*23*). However, it is well established that TCLA X4 HIV-1 strain Envs cannot mediate fusion or fusion is inefficient with primary human macrophages (*3*). Further, it has been observed that a significant proportion of primary X4 HIV-1 isolates cannot enter macrophages (R. Collman, personal communication). In addition, the methods of macrophage differentiation (usage of GM-CSF or LPS) influence CD4 and coreceptor expression (*24–27*), and hence may differentially affect the entry efficiency of both X4 and R5 viruses in *in vitro* assays. Finally, although there are single reports of infected homozygous defective CCR5 patients, it is well established that R5 strains are involved in over 90% of HIV transmissions, while X4 strains appear relatively late in the course of infection (*28*) (reviewed in (*29*)).

In conclusion, the results presented here suggest that inefficient formation of CD4–CXCR4 and gp120–CD4–CXC4 complexes does play an important role for the mechanism of cell resistance to HIV-1 Env-mediated fusion and may have important implications for the mechanism of macrophage resistance to entry of X4 HIV-1 strains *in vivo.*

Materials and Methods. Cells, vaccinia viruses, gp120, sCD4, and antibodies. Human monocytes and lymphocytes were obtained from peripheral blood. Monocyte-derived macrophages were prepared by countercurrent centrifugation elutriation of PBMCs and differentiation as previously described (*30*). The vaccinia viruses used to overexpress CD4 (vCB3) and CXCR4 (vCBYF1) were previously described, respectively, in (*31, 32*). The vaccinia virus for CCR5 (vvCCR5-1107) was developed by using the CCR5 cDNA from pCDNA3 (provided by M. Parmentier) which was subcloned into the *Sma*I site of pMC1107 (*33*) by *Bam*HI–*Xba*I restriction and blunt-end cloning into the *Sma*I site. The vaccinia viruses expressing different HIV-1 Envs were previously described (*3*). The HIV Env gp120 was produced by co-infection of BS-C-1 cells (ATCC CCL26) with vaccinia virus recombinant vPE6 via the hybrid vaccinia virus–T7 system (*34*) with a multiplicity of infection (m.o.i.) of 10 PFU/cell under serum-free media (OPTI-MEM, Life Technologies, Gaithersburg, MD) conditions and purified from the culture supernatants 30 h postinfection by affinity chromatography using lentil lectin–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (*35*). Soluble CD4 (sCD4) and the anti-CD4 polyclonal antibody T4-4 were obtained through the AIDS Research and Reference Reagent Program from R. Sweet (SmithKline and Beechman Pharmaceuticals). The anti-CD4 mAb OKT4 was purchased from Ortho Diagnostic. The anti-CCR5 mAb m182 was purchased from R&D Systems (Minneapolis, MN). The anti-CXCR4 mAb 12G5 was a kind gift from J. Hoxie (University of Pennsylvania, Philadelphia, PA). The anti-CXCR4 mAb 4G10 was raised against a peptide from the CXCR4 N-terminus.

Immunoprecipitation. Cells (typically 5–10 $\times$ 10^6 per sample) were washed once with phosphate-buffered saline (PBS) and then resuspended in PBS at a final density of 10^7/ml. Immunoprecipitating antibodies at the required concentration, typically 1.5–3 μg/ml, were added to the cell suspension and incubated with gentle mixing for 1 h at 37°C. Cells were then pelleted by centrifugation and resuspended in lysis buffer (1% Brij97, 5 mM iodoacetamide (added immediately before use), 150 mM NaCl, 20 mM Tris (pH 8.2), 20 mM EDTA, and protease inhibitors) at 4°C for 1 h with gentle mixing. The nuclei were pelleted by centrifugation at 14,000 rpm for 25 min in a refrigerated Eppendorf centrifuge. Protein G–Sepharose beads (Sigma, St. Louis, MO) prewashed with PBS were added to the samples and incubated at 4°C for 14 h. The beads were then washed four times with 1 ml of ice cold lysis buffer. Samples were then eluted by adding 4$\times$ sample buffer for SDS–PAGE gels and boiled for 5 min or left overnight at 37°C. They were run on a 10% SDS–PAGE gel and were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 20 mM Tris–HCl (pH 7.6) buffer containing 140 mM NaCl, 0.1% Tween 20, and 5% nonfat powdered milk. These membranes were incubated with the respective antibodies, then washed, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, and developed by using the supersignal chemiluminescent substrate from Pierce (Rockford, Il).

Flow Cytometry. Cells (typically 0.5 mln) were incubated for 1 h on ice with the antibodies (10 μg/ml), then the cells were washed and incubated for another hour on ice with rabbit IgG (10 μg/ml) (Sigma, St. Louis, MO), and then washed and incubated for 1 h with an anti-mouse phycoerythrin-conjugated polyclonal antibody (Sigma). The cells were washed and fixed with paraformaldehyde on ice for 10 min. The flow cytometry measurements were performed with FACSaliber (Becton–Dickinson, San Jose, CA). Calibrating beads with a known number of phycoerythrin molecules were purchased (Becton–Dickinson) and used for calibration of the signal intensity.

The number of receptor molecules was approximately estimated from the known signal intensity by using the equation describing the intensity of the calibrating beads, assuming that each antibody molecule is conjugated on average with two phycoerythrin molecules and binds to two receptor molecules.

Cell Fusion Assay. The cell–cell fusion assay was previously described (*36*). The extent of fusion was quantitated colorimetrically.

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